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# EFFECTS OF JUVENILE HORMONE ON ECDYSONE-DEPENDENT DEVELOPMENT IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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Regulation of ecdysone production by insect prothoracic glands (PG) is generally considered to be the province of the brain and its prothoracicotropic hormone (PTTH). The insect life cycle is often viewed as a series of hormonally silent periods punctuated by bursts of PTTH secretion which drive production of ecdysone by the PG, thereby triggering molting and metamorphosis. Juvenile hormone (JH) is generally considered to have primarily morphogenetic effects which are most often manifest as a retardation of metamorphosis with maintenance of the *status quo*.

In addition to this role, JH is known to exert effects on the rate of ecdysone-dependent events in some systems. JH has long been known to be capable of provoking the development of diapausing saturniid pupae (Williams, 1959; Gilbert and Schneiderman, 1959). Hiruma, Shimada, and Yagi (1978) described a similar tropic effect of JH on the development of neck-ligated mature larvae and of brainless pupae in the noctuid *Mamestra brassicae*. In all these cases the authors concluded that JH exerted its stimulatory effect on the PG. By contrast, Nijhout and Williams (1974) demonstrated that JH delayed the onset of wandering in intact mature fifth-instar larvae of *Manduca sexta*. This result was interpreted as an inhibitory effect of JH on the release of PTTH by the brain, thus indirectly blocking ecdysone secretion by the PG.

In the present study we sought to clarify these seemingly contradictory effects of JH by further examination of the actions and interactions of the brain, the corpora allata (CA), and the PG in the postembryonic development of *Manduca*. The findings presented here direct attention to a previously unsuspected shifting role for JH in the control of ecdysone's secretion, metabolism, or action.

## Materials and Methods

Larvae were reared at 25° C on an artificial diet, as described by Bell and Joachim (1976) and Truman (1972), under either short-day (SD, 12 hr light : 12 hr dark) or long-day (LD, 17 hr light : 7 hr dark) photoperiods. Time of day was arbitarily referenced to lights-off at midnight (24 : 00 = 00 : 00). Larvae of the following types were segregated early in the photophase of each day : freshly ecdysed fourth-instar larvae and pharate fourths showing slipped head capsules, freshly ecdysed fifths, and fifths showing freshly exposed dorsal vessels. The timing of events in the life cycle was as described by Truman (1972) and Truman and Riddiford (1974) except that the first 24 hr of each stage was termed Day 1 rather than Day 0.

Neck ligatures were placed between the head and prothorax of larvae and between the head and prothoracic spiracles of pupae within 6 hr after pupal ecdysis. Isolated abdomens of larvae were obtained by placing ligatures across the first

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abdominal segment or between the metathorax and first abdominal segment. In all cases the portion anterior to the ligature was excised. Ligatures were of cotton-covered polyester (J. & P. Coats). Individuals were anesthetized with $CO_2$ prior to ligation and the surgical procedures.

Operations other than ligation were carried out on individuals immersed in insect Ringer's solution (Ephrussi and Beadle, 1936). Manipulations of the larval brain were performed through a small flap cut to one side of the midline just above the juncture of the cuticular sutures of the head capsule. The brain was easily located and gently drawn toward the incision by the entering bundles of trachea and nerves. These bundles were severed with micro-scissors and the brain withdrawn. Pupal brains were removed through a horizontal incision on the vertex of the head. All surgical incisions were sealed by apposition of the cut edges, thorough blotting of the region surrounding the incision, and application of a small drop of melted Tackiwax (Cenco). Less than 5% of individuals failed to survive the operations, and these typically died within 1–3 days. They have been excluded from the data presented.

The JHA "Hydroprene" (Zoecon, ZR-512) was used in the experiments described; all major findings were confirmed and duplicated with C18-JH (JH-1) and the JHA "epoxygeranyl sesamol" (both from Eco-Control). Hormones were dissolved in reagent grade acetone and applied to the dorsal thorax with a 100 $\mu$l Hamilton syringe on a repeating dispenser.

## Results

### *Effects of JH on the metamorphsis of neck-ligated larvae*

Once *Manduca* larvae in the fourth or fifth instars have attained a critical weight peculiar to each instar, they are able to develop following neck ligation (personal observations). Whenever such development takes place, it consists not of a larval molt such as one would anticipate of the fourth instar, but of exposure of the dorsal vessel—a distinctive prodrome of pupation. Indeed, those individuals surviving for a week or more after heart exposure usually proceed to form headless pharate pupae which in some cases undergo successful ecdysis.

A homogeneous group of Day-3, second-gate, fourth-instar larvae were neck-ligated at 20 : 00–21 : 00 and treated with JHA at doses ranging from 0.0001–1 $\mu$g. As shown by the hatched line in Figure 1, individuals receiving subthreshold doses exposed their dorsal vessels after an average of 4 days. Above the threshold dose of 0.05 $\mu$g, the delay was proportional to the logarithm of the dose. In additional experiments we found that neck-ligated fourths receiving 2.5 $\mu$g or more never exposed their dorsal vessels, nor did they show any other sign of development during their 1–2 weeks of survival.

The effect of JHA application was next examined in a group of second-gate LD fifth-instar larvae which were neck-ligated at 20 : 00–22 : 00 on Day 4. Here again (Fig. 1), application of JHA in doses above a critical level (in this case *ca.* 0.5 $\mu$g) delayed exposure of the dorsal vessel. The highest dose tested delayed heart exposure about 10 days beyond controls. In a further experiment not shown in Figure 1, daily application of 10 $\mu$g of JHA to similar neck-ligated larvae prevented any further development during their 3 to 4 weeks of survival.

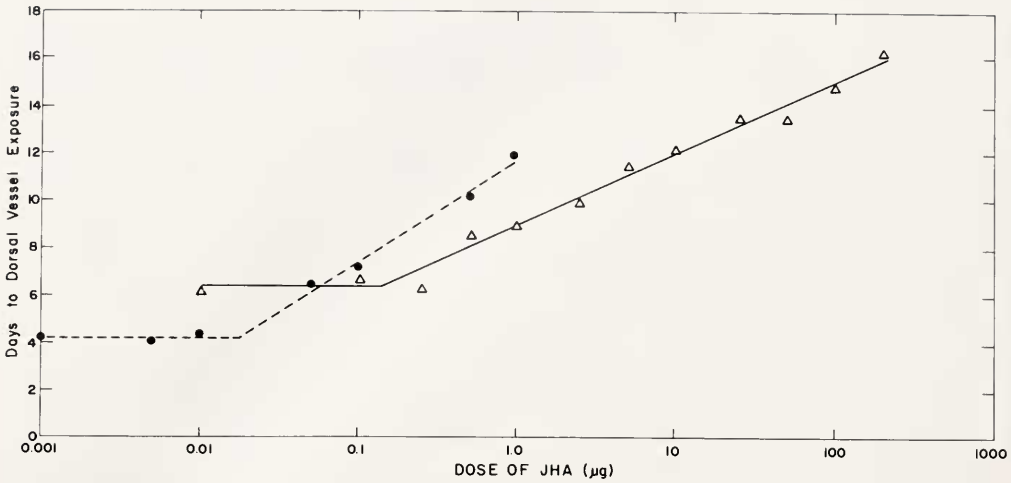


FIGURE 1. Effects of JHA application on the time to dorsal-vessel exposure of neck-ligated fourth- (closed circles) and fifth- (open triangles) instar larvae. Fourth-instar larvae weighed 0.75–1.0 g and were ligated between 20:00 and 21:00 AZT. Fifth-instar larvae weighed 7.0–8.5 g and were ligated between 20:00 and 22:00 AZT on Day 4 of the instar. Each point represents an average of the time to dorsal-vessel exposure of those 12–25 larvae which eventually exposed the dorsal vessel. At the higher concentrations some larvae in both instars died from starvation and desiccation prior to dorsal-vessel exposure. Standard deviations ranged from ±1 to ±2.5 days and tended to be larger at the higher doses.

*Effects of JH on the development of brainless larvae*

We have previously observed (Safranek and Williams, 1980) that when the brains of second-gate fourth-instar larvae were extirpated prior to 23:00 on Day 3, none of the brainless larvae showed any development during the remaining 1.5–3 weeks of survival. The same was true when the experiment was repeated on a similar group of 20 brainless fourths which received, in this case, daily applications of 20 μg JHA following brain removal. Yet when another group of brainless larvae were neck-ligated several days after brain removal, many of the individuals exposed the dorsal vessel within a few days. This could be prevented by application of a single dose of 20 μg JHA immediately after neck ligation.

Similar experiments were performed on mature fifth-instar larvae (second-gate Day-4 LD larvae weighing 7.0–8.5 g at 19:00–23:00) approximately 30 hr before they would have undergone dorsal vessel exposure and initiated wandering behavior. The brains were removed from 36 larvae and another 15 were sham-operated. Each individual was then placed in a plastic container without food. Eighteen of the brainless larvae received daily applications of 2 μl of acetone. These all initiated wandering after 4.1 ± 1.7 (standard deviation) days. The other 18 brainless larvae as well as the 15 sham-operated larvae received daily applications of 20 μg JHA. The sham-operated larvae all molted to sixth-instar larvae 5.4 ± 1.1 days following operation. By contrast, the JH-treated brainless larvae never undertook development of any kind although they survived for 21.0 ± 7.1 days. Additional experiments demonstrated that as little as a single dose of JHA administered to brainless fifths could provoke a substantial delay in metamorphosis. For example, topical application of 200 μg of JHA immediately after

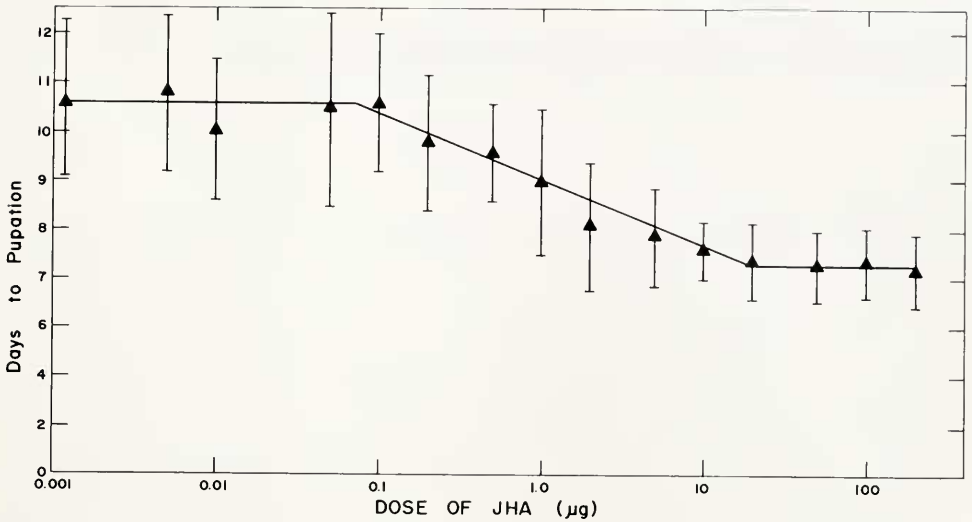


FIGURE 2. Effects of JHA application on the time to pupation of neck-ligated wandering larvae. Larvae were ligated between 07:00 and 12:00 AZT on the first day of wandering. All groups contained roughly equal numbers of first- and second-gate larvae. Points represent averages of the times to pupation of 15–20 larvae. Larvae which died prior to pupation were not included. This mortality, which ranged up to 30%, was highest at the lowest concentrations shown. Vertical bars equal ± standard deviation.

removal of the brain as late as 12:00 on Day 5 of the final instar often delayed the onset of dorsal vessel exposure by more than a week.

*Effects of JH on neck-ligated and brainless wandering larvae*

Up to this point we have considered the effects of neck ligation or brain removal, with or without JH treatment, on the initiation of metamorphosis as signaled by exposure of the dorsal vessel. We carried out the same experimental maneuvers on fifth-instar larvae which had already exposed their dorsal vessels and initiated the 2-day period of wandering. The effects were judged in each case by the time which elapsed between the treatment and the completion of pupal development, which was indicated by the formation of a tanned pupal cuticle.

In normal larvae reared at 25° C, an average of 5 days elapses between the onset of wandering and pupal ecdysis. This period was extended to an average of 10–11 days when neck ligation was carried out on the first day of wandering. Specific doses of JHA were applied immediately after neck ligation on the first day of wandering. The results, summarized in Figure 2, show that the delay occasioned by neck ligation was shortened in a dose-dependent manner. However, neither the highest single dose tested nor doses repeated daily were able to eliminate completely the delay resulting from neck ligation.

Substantially the same result was observed in parallel experiments carried out on larvae that had been neck-ligated on the second day of wandering (data not shown). Neck ligation after the second day of wandering caused little or no delay relative to unligated controls—a finding that suggests that the head's contribution to pupation is completed during the second day of wandering. Larval abdomens isolated by ligation on the first day of wandering survived for 1–3 weeks but showed

TABLE I

*Effect of various procedures on the pupation of wandering-stage short-day larvae.*

| Procedure | Number of larvae | Average time to pupation (days ± s.d.) |
|---|---|---|
| No treatment | 16 | 4.8 ± 0.2 |
| JHA application | 17 | 4.5 ± 0.2 |
| Sham head operation | 17 | 5.3 ± 0.2 |
| Sham head operation and JHA application | 17 | 4.9 ± 0.5 |
| Brain removal | 17 | 6.8 ± 0.7 |
| Brain removal and JHA application | 17 | 5.7 ± 0.3 |
| Neck ligation | 25* | 10.3 ± 1.6 |
| Neck ligation and JHA application | 22 | 6.3 ± 0.5 |

* Four additional larvae died beyond 10 days after undergoing apolysis without apparent cuticle formation.

no further development. In the absence of the thorax, these abdomens could not be rescued even by large or repeated doses of JHA.

Removal of the brain from wandering larvae causes a delay of about 2 days in the onset of pupation (Safranek and Williams, 1980). Preliminary experiments suggested that JH might lessen this delay. Accordingly, a large number of synchronous Day–1 SD wandering larvae derived from a group of second-gate fifth instars were subjected to a variety of procedures to examine the effects of JHA application. All procedures were performed between 12:00 (Hour 0 for purposes of calculation) and 15:00. Individuals receiving JHA were treated with 200 μg of JHA immediately after ligation as well as on each of the subsequent 2 days. Because of the relatively small differences anticipated between groups, larvae were examined for pupation every 6 hr. The results of this experiment (Table I) show that the addition of JHA accelerated the pupation of all groups—even intact larvae. JHA reduced but did not eliminate the delay provoked by brain extirpation. Furthermore, the JHA treatment eliminated most of the very long delay induced by neck ligation.

Larvae treated with JHA after the onset of wandering ultimately formed normal pupal cuticle. This was anticipated on the basis of results of earlier experiments (Truman, Riddiford, and Safranek, 1974) which demonstrated the inability of JH to disturb normal pupal formation when administered after the initiation of wandering. So also, the morphology of pupae developing from brainless larvae was normal. Though some of the individuals failed to undergo ecdysis, this was apparently due to abnormalities in the head region secondary to the earlier operation. Many of the JHA-treated larvae formed healthy, intact headless pupae which ecdysed successfully and lived for several weeks. Even those which failed to ecdyse formed normal, well-tanned pupal cuticles. By contrast, headless larvae which did not receive JHA treatment eventually formed pupal cuticles which were recognizably pupal but otherwise thin, fragile, and often poorly tanned. Those few which successfully ecdysed had short wings and rarely survived more than a few days. Many of these abnormalities appeared to be the result, not so much of the absence of JH, as of the delay which resulted from the lack of JH. When untreated head-

less wanderers were kept in an environment whose atmosphere was saturated with water vapor, cuticular abnormalities seen previously were markedly reduced although the onset of pupation was still considerably delayed.

*Effects of JH on the development of headless and brainless pupae*

In a series of preliminary experiments, non-diapausing pupae were neck-ligated within 6 hr after ecdysis. Though these decapitated individuals failed to enter the expected extended diapause, their development was nevertheless always delayed by at least 2 weeks.

To examine the effect of JH, we decapitated several hundred potentially non-diapausing, recently pupated LD pupae and then applied a specific dose of JHA to each individual. The development of the treated populations was examined after two weeks. As shown in Figure 3, the JHA treatment accelerated development in a dose-dependent manner. All individuals initiating development within the first 2 weeks showed abnormal characteristics attributable to JH, as described previously by Riddiford and Ajami (1973).

Diapause-destined pupae derived from SD larvae were subjected to brain extirpation within 12 hr after pupation. As was shown by Safranek and Williams (1980), all such individuals typically require at least 8 weeks to initiate development. One week after the operation 25 brainless pupae and 25 1-week-old unoperated diapausing pupae were treated with 200 μg of JHA. When examined 2 weeks later, development had been initiated by 24 of the brainless pupae and 19 of the unoperated controls.

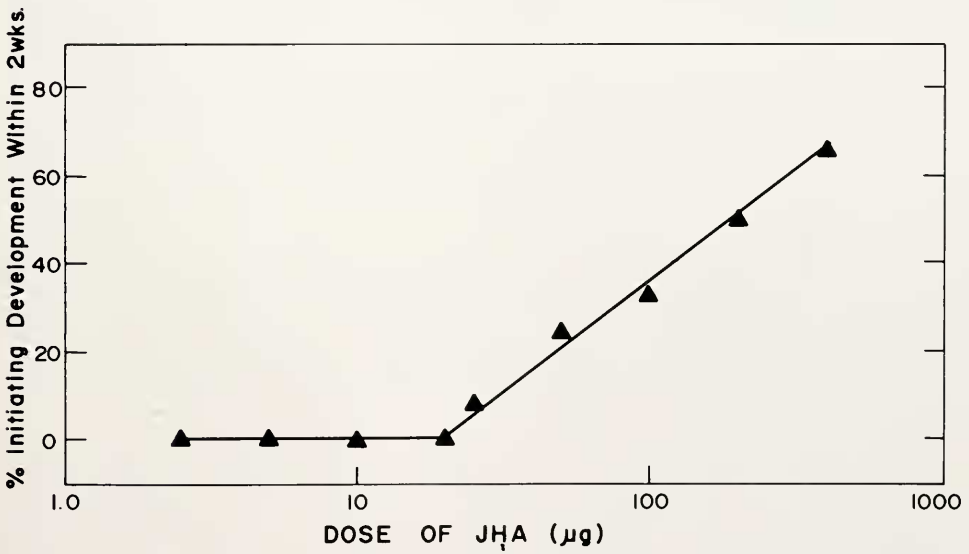


FIGURE 3. Effects of JHA application on initiation of development by neck-ligated pupae. Non-diapausing pupae were ligated within 6 hr after pupation. Initiation of development was recognized by apolysis of the wing epidermis. The time to completion of development from this point did not differ from that seen in unligated controls. Groups of 15–18 pupae were utilized at each dose tested. The data at two weeks are presented because some ligated controls initiated development during the third week. All pupae eventually developed after many weeks.

## Discussion

JH accelerated the development of tobacco hornworms from the very onset of the wandering stage. This was true not only of intact wandering larvae and pupae but of brainless and neck-ligated individuals as well. The report by Hiruma, Shimada, and Yagi (1978) of a similar pattern of response by wandering larvae and pupae of the noctuid *Mamestra brassicae* suggests that the potential for JH stimulation of development may be widespread among mature larval and pupal Lepidoptera.

Mature larvae of the fourth or fifth instar initiate metamorphosis following appropriately timed neck ligation. The most novel finding in the current study is that prior to the onset of the wandering period, as signaled by exposure of the dorsal vessel, the metamorphic development of such neck-ligated larvae is actually delayed or inhibited by JH. Though this effect of JH is lost once the brain has provoked the initiation of a larval molt or of wandering, application of JH to larvae neck-ligated even a few hours before this time was able to delay or completely prevent any subsequent development.

Fifth-instar larvae whose brains were extirpated late in the instar underwent metamorphosis as did their neck-ligated counterparts. And as in the case of neck-ligated larvae, this development could be delayed or prevented by JH application. In contrast to this metamorphic development of brainless fifth-instar larvae, brainless fourths underwent a complete developmental arrest. When the brains were extirpated from fourth-instar larvae which would have exposed the dorsal vessel had they been neck-ligated, the larvae never underwent any type of development. This outcome was not altered by treatment with JH. The developmental inactivity of these larvae therefore resembled that of the neck-ligated larvae treated with higher doses of JH. Indeed, when such brainless larvae were neck-ligated even several days after brain removal, they proceeded to initiate metamorphosis, as signaled by exposure of the dorsal vessel. As with other neck-ligated larvae, this developmental response could be prevented by the application of JH. These observations are consistent with a continued secretion of JH by the CA of brainless fourth-instar larvae. By contrast, the initiation of dorsal–vessel exposure by brainless fifths indicates that the JH in these larvae routinely declines to levels permitting the onset of metamorphosis.

The ability of JH to delay the onset of wandering in intact mature fifth-instar larvae has been previously noted (Nijhout and Williams, 1974). This result was interpreted as an inhibitory effect of JH on the brain's release of PTTH. However, the present study has demonstrated that the pronounced ability of JH first to delay and subsequently to accelerate development persists in brainless individuals. Consequently the brain and its PTTH cannot be the sole targets for these effects of JH, if in fact they are targets at all.

Although isolated abdomens do not respond to JH application, injections of $\alpha$- or $\beta$-ecdysone can cause them to undergo larval molting, dorsal–vessel exposure, pupation, and adult development (Fain and Riddiford, 1976; Nijhout, 1976; personal observations). Ecdysone must therefore be the ultimate vehicle for the various responses of neck-ligated larvae and pupae to JH application. JH might intervene in one or more of the following processes: 1) $\alpha$-ecdysone synthesis and secretion by the PG; 2) conversion of $\alpha$-ecdysone to $\beta$-ecdysone; 3) ecdysone inactivation and excretion; 4) response at the cellular level to ambient ecdysone. These several possibilities will be examined in subsequent communications in this series.

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## Summary

1. Juvenile hormone delayed or prevented the onset of metamorphosis by neck-ligated fourth-instar tobacco hornworm larvae and by fifth instars neck-ligated prior to the cessation of feeding.

2. After the onset of the wandering period JH had precisely the opposite effects in that it accelerated the onset of metamorphosis. This was the case both for wandering larvae and for pupae, irrespective of whether they were intact or subjected to brain removal or neck ligation.

3. These findings point to a previously unsuspected shifting role of JH in the control of metamorphosis.

4. Fourth-instar larvae underwent no further development after brain removal unless they were also effectively allatectomized by neck ligation. Evidently the secretion and inhibitory action of JH persists in brainless fourth-instar larvae.

5. Brainless fifths, by contrast, were often able to initiate metamorphosis despite the continued presence of their CA. Development could be prevented by daily application of JH. The disappearance of effective levels of JH, which normally occurs in the late fifth instar as a necessary prelude to metamorphosis, can evidently take place in the absence of the brain. This implies that the brain is not prerequisite for the decline in JH.

6. Since the ability of JH first to delay and subsequently to accelerate the development of intact individuals also persists after brain removal, the brain and its PTTH cannot be the sole targets for these effects of JH, if in fact they are targets at all.

7. The shifting effects of JH on development appear to be ecdysone-dependent. Four possible sites of JH action are identified.

## LITERATURE CITED

Bell, R. A., and F. G. Joachim, 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. Am.*, **69**: 365–373.

Ephrussi, B., and G. W. Beadle, 1936. A technique of transplantation for *Drosophila*. *Am. Nat.*, **70**: 218–225.

Fain, M. J., and L. M. Riddiford, 1976. Reassessment of the critical periods for prothoracicotropic hormone and juvenile hormone secretion in the larval molt of the tobacco hornworm *Manduca sexta*. *Gen. Comp. Endocrinol.*, **30**: 131–141.

Gilbert, L. I., and H. A. Schneiderman, 1959. Prothoracic gland stimulation by juvenile hormone extracts of insects. *Nature*, **184**: 171–173.

Hiruma, K., H. Shimada, and S. Yagi, 1978. Activation of the prothoracic gland by juvenile hormone and prothoracicotropic hormone in *Mamestra brassicae*. *J. Insect Physiol.*, **24**: 215–220.

Nijhout, H. F., 1976. The role of ecdysone in pupation of *Manduca sexta*. *J. Insect Physiol.*, **22**: 453–463.

Nijhout, H. F., and C. M. Williams, 1974. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol.*, **61**: 493–501.

Riddiford, L., and A. Ajami, 1973. Juvenile hormone: its assay and effects on pupae of *Manduca sexta*. *J. Insect Physiol.*, **19**: 749–762.

Safranek, L., and C. M. Williams, 1980. Studies of the prothoracicotropic hormone in the tobacco hornworm, *Manduca sexta*. *Biol. Bull.*, **158**: 141–153.

Truman, J. W., 1972. Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. Exp. Biol.*, **57**: 805–820.

Truman, J. W., and L. M. Riddiford, 1974. Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.*, **60**: 371–382.

Truman, J. W., L. M. Riddiford, and L. Safranek, 1974. Temporal patterns of response to ecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **39**: 247–262.

Williams, C. M., 1959. The juvenile hormone. I. Endocrine activity of the corpora allata of the adult cecropia silkworm. *Biol. Bull.*, **116**: 323–328.